Journal of Microbiological Methods 77 (2009) 37–40

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of a culture independent method to characterize the chemotactic response of *Flavobacterium columnare* to fish mucus

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ARTICLE INFO

Article history:
Received 21 November 2008
Received in revised form 22 December 2008
Accepted 22 December 2008
Available online 3 January 2009

Keywords:
Chemotaxis
Fish mucus
Flavobacterium columnare

ABSTRACT

Flavobacterium columnare, the causative agent of columnaris disease, is a significant pathogen of many freshwater fish species worldwide and is considered one of the most important pathogens impacting the channel catfish (*Ictalurus punctatus* Rafinesque) industry in the United States. Recent research has demonstrated that *F. columnare* exhibits a chemotactic response to mucus from the skin of catfish and this response may be important for pathogenesis. In this study, a culture independent method was developed to quantify the chemotactic response of *F. columnare* to skin mucus. The method employs the use of blind-well chemotaxis chambers which overcomes difficulties using the traditional capillary tube assay and uses a cell proliferation assay to quantify viable cells which reduces the time and labor associated with culturing the bacterium. Application of the method to two sets of catfish skin mucus samples demonstrated that there is variation in the chemotactic response of *F. columnare* to individual catfish mucus samples, and similar results were obtained to previously published research using the traditional capillary tube method. The use of this method will enhance the ability to further characterize the chemotactic response of *F. columnare* to catfish skin mucus in order to increase the understanding of this important host–pathogen interaction.

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1. Introduction

Flavobacterium columnare is a Gram-negative bacterium that causes columnaris disease in fish. The bacterium is a significant pathogen of wild and cultured fish species due to its broad geographic distribution and an ability to infect most freshwater fish species (Plumb, 1999). *F. columnare* causes external infections characterized by yellow-pigmented necrotic lesions of the gills and skin (Hawke and Thune, 1992; Plumb, 1999). Systemic infections are also common in which there may be no external lesions; however, large numbers of bacteria can be isolated from internal organs (Hawke and Thune, 1992). Columnaris disease is responsible for large economic losses in aquaculture and is one of the most important bacterial diseases impacting the channel catfish (*Ictalurus punctatus* Rafinesque) industry in the United States (Wagner et al., 2002).

Recently it has been demonstrated that *F. columnare* exhibits a chemotactic response toward skin mucus of channel catfish (Klesius et al., 2008). It was suggested that this response may be important for pathogenesis because genomovar II strains, which are highly virulent for channel catfish (Shoemaker et al., 2008), exhibited a significantly higher chemotactic response toward skin mucus compared to less virulent, genomovar I strains. Thus, an increased understanding of the chemotactic response of *F. columnare* and the channel catfish mucus components involved may provide new insights into host–pathogen relationships and novel control strategies.

The method used for the chemotaxis experiments with *F. columnare* in the aforementioned research was a slight modification of the capillary tube method described by Alder (1973), which is the typical method used for such studies. Although this method has been used successfully in our laboratory, we have found it to be limiting in two ways. Firstly, the use of the capillary tubes can be tedious and difficult as reported by others (Han and Cooney, 1993; Mazumder et al., 1999). Secondly, in order to quantify bacterial chemotaxis following an experiment, culture of the bacteria is required. This is relatively easy to perform; however, it can be labor and time intensive due to the requirement of preparing dilutions, plating volumes of each dilution onto replicate solid bacteriological media, and incubating the bacteria for a sufficient amount of time to allow for colony growth. This is especially the case when it is desired to perform experiments utilizing a number of different treatments and also when a fastidious microorganism, such as *F. columnare*, is used, in which 48–72 h is required for colony development (Cain and LaFrentz, 2007). Under these circumstances, it may take 3–4 d to obtain the results from a chemotaxis experiment. Therefore, the objective of this study was to develop a simple, rapid, and culture independent method to quantify the chemotactic response of *F. columnare* toward catfish skin mucus.

2. Materials and methods

2.1. Bacterial culture

A virulent strain of *F. columnare* (ALG-00-530) was used for all portions of this study. This strain was originally isolated in 2000 from

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0167-7012/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.mimet.2008.12.011

a diseased channel catfish in Alabama (USA) and belongs to genomovar II (Arias et al., 2004). Stock suspensions of the strain were maintained at −80 °C in 20% glycerol and were used to inoculate cultures. The bacterium was cultured in 125 mL flasks containing 25 mL of modified Shieh broth (0.5% tryptone, 0.2% yeast extract, 45.6 μM $CaCl_2 \cdot 2H_2O$, 1.1 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 3.6 μM $FeSO_4 \cdot 7H_2O$, pH 7.2) for 24 h at 28 °C on an orbital shaker set at 90 rotations per minute.

For the cell proliferation assay to quantify *F. columnare* in culture, a 24 h culture of *F. columnare* was adjusted to an optical density of 1.0 at 540 nm. The number of viable colony forming units (cfu) mL^{-1} was determined by dispensing 50 μL of serial dilutions (in duplicate) onto Shieh agar plates using a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA, USA). Plates were incubated for approximately 72 h at 28 °C and colonies were counted to enumerate the cfu mL^{-1} using standard procedures. The *F. columnare* cultures were prepared identically for the chemotaxis assays.

2.2. Cell proliferation assay to quantify F. columnare in culture

The ability of the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) to quantify viable cells of *F. columnare* was determined. The adjusted *F. columnare* culture (optical density of 1.0 at 540 nm) was serially diluted (in doubling dilutions) from 1:10 to 1:640 using modified Hanks' balanced salt solution (HBSS; Sigma, St. Louis, Missouri, USA). Following dilution, 100 μL of each dilution or HBSS alone (in duplicate) were added to a 96-well plate and then 20 μL of the combined MTS/PMS (MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PMS, phenazine methosulfate) solution was added to each well. The plate was covered to protect from light, incubated for 4 h at 28 °C, and then the absorbance at 490 nm was recorded using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. Collection of mucus

Channel catfish (NWAC-103) reared at the USDA-ARS Aquatic Animal Health Research Unit (Auburn, AL, USA) were used for mucus collection. Fish ranged in length from approximately 20–30 cm and were maintained using standard husbandry practices in a 340 L fiberglass tank supplied with 25–29 °C de-chlorinated municipal water with supplemental aeration. Individual catfish were anaesthetized with approximately 100 mg L^{-1} tricaine methanesulfonate (Argent Chemicals, Redmond, WA, USA). Following anaesthetization, individual fish were held vertically and mucus was gently scraped from the skin surface into a petri dish with a cell scraper. Mucus was collected from the petri dish and placed into 5 mL centrifuge tubes. Mucus samples were centrifuged for 30 min at 5000×g to remove particulate material and the supernatant was harvested and frozen at −20 °C. The protein concentration of each mucus sample was determined using a Micro BCA™ Protein Assay (Pierce, Rockford, IL, USA). A pool of mucus was collected from 5 individual fish for use in validating the chemotaxis assay. Additionally, two sets of samples were collected, each consisting of eleven mucus samples from individual fish; set one corresponds to mucus samples 1–11 and set two corresponds to mucus samples A–K. Ten microliters of each mucus sample were streaked for isolation onto tryptic soy agar and Shieh agar plates and incubated at 28 °C for 72 h to check for bacterial contamination.

2.4. Chemotaxis assay

Chemotaxis assays were performed using blind-well chemotaxis chambers (Corning CoStar, Cambridge, MA, USA). The assay was initially validated using a pool of mucus from five fish and the protein concentration was adjusted to 0.1 and 0.05 μg μL^{-1}. Subsequently, the assay was applied to both sets of mucus samples collected, in which the protein concentrations were adjusted to 0.1 and 0.2 μg μL^{-1} for set one and set two, respectively.

The bottom wells of the chambers were filled with 200 μL of the diluted mucus samples or HBSS as a negative control (duplicate chambers per sample). Nuclepore® polycarbonate membranes (Whatman, Florham Park, NJ, USA) with a diameter of 13 mm and pore size of 0.8 μm were carefully placed over the top of the bottom wells with the shiny side facing up. Following assembly of the chambers, 200 μL of the adjusted *F. columnare* culture (optical density of 1.0 at 540 nm) was placed into the top wells of each chamber and incubated for 1 h at room temperature. After incubation, the contents of the top wells were removed using a pipette, the chambers were disassembled, and the membranes were carefully removed. The contents of the bottom well from each chamber were mixed, and 100 μL were removed and placed into a flat bottom Microtiter® 96-well plate (Thermo Scientific, Milford, MA, USA). Each diluted mucus sample or HBSS alone was added to the 96-well plate (100 μL) in duplicate to determine the background absorbance due to the sample alone. Positive controls, consisting of 100 μL of the adjusted *F. columnare* culture diluted 1:5 in HBSS (in duplicate), were also added to the 96-well plate. Following addition of samples to the 96-well plate, 20 μL of the combined MTS/PMS solution was added to each well and mixed. The plate was covered to protect from light, incubated for 4 h at 28 °C, and then the absorbance at 490 nm was recorded using a Model 680 microplate reader (Bio-Rad). Four independent chemotaxis assays were performed using a pooled mucus sample for the validation of the assay, and three or four independent chemotaxis assays were performed using the mucus samples from set one or set two, respectively.

2.5. Data analysis

For the cell proliferation assay to quantify *F. columnare* in culture, absorbance values obtained from each dilution of the *F. columnare* culture were corrected to remove background absorbance by subtracting the mean absorbance value obtained from the HBSS only control. The mean corrected absorbance values for each dilution were plotted against the corresponding number of viable *F. columnare* cfu $well^{-1}$. Linear regression was performed using GraphPad Prism (version 2.01, GraphPad Software, San Diego, CA, USA) to determine the correlation between the corrected absorbance at 490 nm and the number of viable *F. columnare* cfu $well^{-1}$.

For the chemotaxis assays, absorbance values obtained from the chemotaxis chambers (test mucus samples and HBSS controls) were corrected to remove background absorbance due to the sample alone.

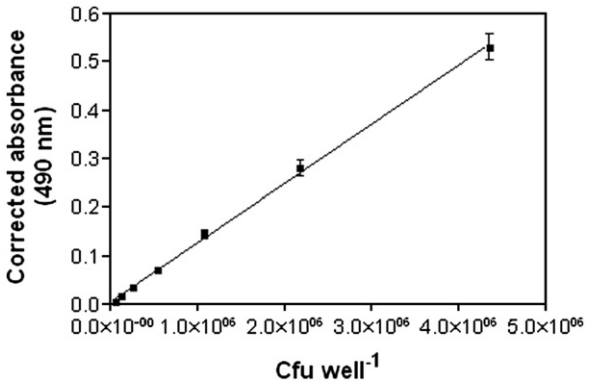

Fig. 1. Correlation ($r^2 = 0.9939$) between viable *F. columnare* (ALG-00-530) cells (cfu $well^{-1}$) and the corrected absorbance at 490 nm measured using the CellTiter 96® AQ_{ueous} Assay. Each data point represents the mean corrected absorbance from duplicate wells containing different numbers of *F. columnare* cells as described in the Materials and methods.

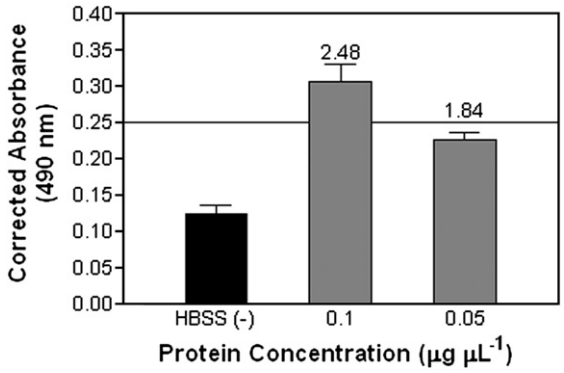

Fig. 2. Mean corrected absorbances at 490 nm measured with the CellTiter 96® AQ_{ueous} Assay following four independent chemotaxis assays using *F. columnare* (ALG-00-530) and a pool of mucus from the skin of five individual catfish at protein concentrations of 0.1 and 0.05 µg $µL^{-1}$ or HBSS controls. The solid line indicates a RCI of 2.0 and the RCI's determined for each protein concentration of mucus is indicated above each bar. Error bars represent the standard error of the mean.

This was performed by subtracting the mean absorbance value of the sample alone from the absorbance values obtained from the duplicate chemotaxis chambers of the corresponding sample. The relative chemotaxis index (RCI) of each individual mucus sample was calculated using the following equation: [mean corrected absorbance of test mucus] / [mean corrected absorbance of HBSS control]. A RCI of greater than 2.0 was considered significant (Alder, 1973; Moulton and Montie, 1979).

3. Results and discussion

There are a number of assays currently available to quantify the number of viable cells in a sample. Initially, a fluorescent cell viability assay was tested and it was found that a high level of background fluorescence occurred in mucus samples alone (data not shown), likely due to the presence of ATP, which was the target for the assay. Therefore, the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was chosen for subsequent testing due to its ease of use. This colorimetric assay is composed of a tetrazolium salt, MTS, and an electron coupling reagent, PMS. MTS is reduced to formazan by living cells and the absorbance obtained is directly proportional to the number of viable cells in a sample (Barltrop et al., 1991). The ability of the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) to quantify viable *F. columnare* cells was assessed and the results demonstrated that there was a positive linear correlation between the corrected absorbance at 490 nm and the number of viable *F. columnare* cells (cfu $well^{-1}$) (Fig. 1). A coefficient of determination (r^2) of 0.9939 was obtained, indicating that the absorbance is a strong predictor of the number of viable *F. columnare* cells.

To overcome the difficulties associated with the use of capillary tubes, blind-well chemotaxis chambers were used. These are simple and easy to use devices that have been used to assess chemotaxis of macrophages (Klesius and Sealey, 1996; Wiedenmayer et al., 2008) and similar chambers have been used for bacteria (van der Marel et al., 2008). The use of the chemotaxis chambers and the cell proliferation assay to quantify the chemotactic response of *F. columnare* was first tested using a pool of catfish skin mucus. Four independent assays were performed using the pooled mucus sample at 0.1 and 0.5 µg protein $µL^{-1}$. Following the chemotaxis assay, the number of *F. columnare* cells that migrated into the mucus samples was quantified using the cell proliferation assay and also by plating dilutions of the bottom well contents onto Shieh agar in order to determine the concordance between both methods. The results of the cell proliferation assay demonstrated that *F. columnare* exhibited a significant chemotactic response towards mucus at a protein concentration of 0.1 µg $µL^{-1}$ with a mean RCI of 2.48 (Fig. 2). The chemotactic response of *F. columnare* was lower at a mucus protein concentration of 0.05 µg $µL^{-1}$ (RCI of 1.84) suggestive of a dilution effect of the chemoattractants in the mucus. Calculation of the RCI's based on the cfu mL^{-1} obtained by plating dilutions of the migrated *F. columnare* resulted in similar values with RCI's of 2.1 and 1.7 for the protein concentrations of 0.1 and 0.05 µg $µL^{-1}$, respectively (data not shown). The agreement of results between both quantification methods demonstrated that the cell proliferation assay is well suited as a non-culture based method to quantify the chemotaxis of *F. columnare* to fish mucus.

Following validation, the assay was applied to the mucus samples from set one (#1–11). The protein concentrations of these ranged from 0.24 to 0.88 µg $µL^{-1}$ and all were culture negative following plating onto TSA and Shieh agar. Each mucus sample was adjusted to 0.1 µg $µL^{-1}$ and three independent chemotaxis assays were performed. The results demonstrated that at this protein concentration, these mucus samples were weakly attractant for *F. columnare* with RCI's of less than 2.0 (Fig. 3). The exception to this was mucus sample #1 in which *F. columnare* exhibited a significant chemotactic response with a RCI of 2.6. This suggested that the chemoattractant concentration in the mucus was too dilute to stimulate a chemotactic response by *F. columnare*. The protein concentration of 0.1 µg $µL^{-1}$ was chosen because this concentration with the pooled mucus sample used for the validation of the assay was demonstrated to be chemoattractive to *F. columnare*. Since there is variability in the chemotactic response of *F. columnare* to mucus from individual fish (Fig. 3), it is likely that at least one of the fish used to produce the pool of mucus contained a high concentration of chemoattractants involved in this response.

A second set of mucus samples (set two, #A–K) was collected to test higher protein concentrations. The protein concentrations ranged from 0.2 to 0.3 µg $µL^{-1}$ and all samples were culture negative following plating onto TSA and Shieh agar plates. Four independent chemotaxis assays were performed using these mucus samples at a protein concentration of 0.2 µg $µL^{-1}$. The RCI's ranged from 0.8 to 3.3 and *F. columnare* exhibited a significant chemotactic response towards 8 of the 11 mucus samples with RCI's greater than 2.0 (Fig. 4). The RCI's obtained in the present study are lower than those reported by Klesius et al. (2008) using the traditional capillary tube method and enumerating viable cell numbers by culture. Although a direct comparison of the two methods was not performed in this study, the results obtained are similar and also demonstrate variation in the chemotactic response of *F. columnare* to mucus samples from individual fish. Klesius et al. (2008) reported a significant chemotactic response of *F. columnare* to 60% of the mucus samples analyzed, whereas this occurred in 73% of samples in the present study. The reason for this variation is not known, but is likely due to differences

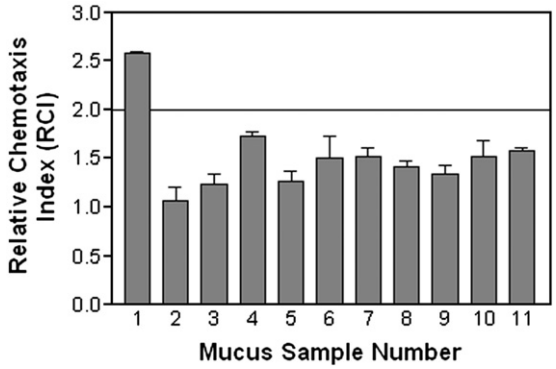

Fig. 3. Chemotactic response of *F. columnare* (ALG-00-530) to mucus from the skin of individual catfish (set one; designated 1–11) at a protein concentration of 0.1 µg $µL^{-1}$. The mean RCI's were calculated following three independent chemotaxis assays as described in Materials and methods. RCI values above the solid line (>2.0) are considered significant. Error bars indicate the standard error of the mean.

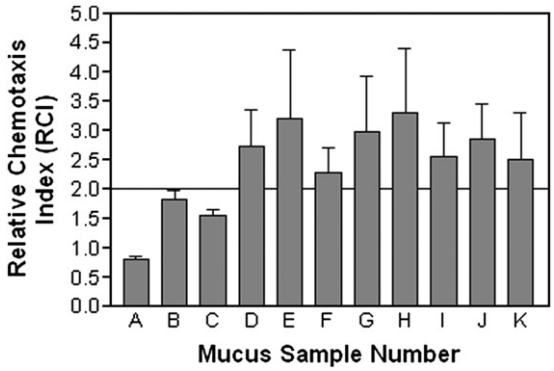

Fig. 4. Chemotactic response of *F. columnare* (ALG-00-530) to mucus from the skin of individual catfish (set two; designated A–K) at a protein concentration of 0.2 μg μL^{-1}. The mean RCI's were calculated following four independent chemotaxis assays as described in Materials and methods. RCI's above the solid line (>2.0) are considered significant. Error bars indicate the standard error of the mean.

in the concentrations of chemoattractants in the mucus from individual fish. Research has demonstrated variation in the concentrations of different compounds found in intestinal mucus from different fish (O'Toole et al., 1999) and such variation is also likely to occur in skin mucus.

In summary, a simple, rapid, and culture independent method has been developed which will enhance the ability to further characterize the chemotactic response of *F. columnare* to fish mucus. The assay overcomes difficulties encountered using capillary tubes and eliminates the time and labor associated with bacterial plating. Research is currently underway to identify the chemoattractant(s) in the mucus of channel catfish and to identify the bacterial factors involved in chemotaxis. Such research will increase the understanding of this host–pathogen relationship and may identify novel control strategies for the prevention of columnaris disease.

Acknowledgements

The authors thank P. Mumma of USDA-ARS for her technical assistance and C. Day and J. McVicker of USDA-ARS for animal care. This research was funded by USDA-ARS CRIS Project No. 6420-32000-022-00D (Aquatic Animal Diagnostics, Pathogenesis and Applied Epidemiology). Mention of trade name or commercial products in this publication is solely for the purpose of providing specific information and does not imply endorsement by the United States Department of Agriculture.

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